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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/67, 15/62, 15/85
A61K 31/70, 41/00

A1

(11) International Publication Number: WO 94/06916

(43) International Publication Date: 31 March 1994 (31.03.94)

(21) International Application Number: PCT/US93/08432

(22) International Filing Date: 8 September 1993 (08.09.93)

(30) Priority data:
07/943,812 11 September 1992 (11.09.92) US

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(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: GENE TRANSCRIPTION AND IONIZING RADIATION: METHODS AND COMPOSITIONS

(57) Abstract

The present invention provides a DNA molecule comprising a radiation responsive enhancer-promoter operatively linked to an encoding region that encodes at least one polypeptide. An encoding region can comprise a single encoding sequence for a p lypeptide or two or more encoding sequences encoding DNA binding, activation or repression domains of a transcription factor. Processes for regulating polypeptide expression and inhibiting tumor growth using such DNA molecules are also provided.

GENE TRANSCRIPTION AND IONIZING RADIATION: METHODS AND COMPOSITIONS

The present application is a continuation-in-part of United States Patent Application Serial Number 07/633,626, filed December 20, 1990, the disclosure of which is incorporated herein by reference.

This invention relates to methods and compositions relating to the regulation of gene transcription and polypeptide expression by ionizing radiation.

Certain genes may play a role in the cellular response to stress or DNA-damaging agents. For example, metallothionein I and II, collagenase, and plasminogen activator are induced after UV irradiation (Angel, et al., 1986; 1987; Fornace, et al., 1988a and b; Miskin, et al., 1981). B2 polymerase III transcripts are increased following treatment by heat shock (Fornace, et al., 1986; 1989a). Furthermore, although the level of DNA polymerase β mRNA is increased after treatment with DNA-damaging agents, this transcript is unchanged following irradiation, suggesting that specific DNA-damaging agents differentially regulate gene expression (Fornace, et al., 1989b). Protooncogene c-*fos* RNA levels are elevated following treatment by UV, heat shock, or chemical carcinogens (Andrews, et al., 1987; Hollander, et al., 1989a). In this regard, the relative rates of *fos* transcription during heat shock are unchanged, suggesting that this stress increased c-*fos* RNA through posttranscriptional mechanisms (Hollander, et al., 1989b).

Investigations of the cytotoxic effects of ionizing radiation has focused on the repair of DNA damage or the modification of radiation lethality by hypoxia (Banura, et al., 1976; Moulder, et al., 1984). In prokaryotes and lower eukaryotes, ionizing radiation has been shown to induce expression of several DNA repair genes (Little, et al., 1982); however, induction of gene expression by ionizing radiation has not been described in mammalian cells. DNA-damaging agents other than x-rays induce expression of a variety of genes in higher eukaryotes (Fornace, et al., 1988, 1989; Miskin, et al., 1981).

What is known about the effects of ionizing radiation is that DNA damage and cell killing result. In many examples, the effects are proportional to the dose rate. Ionizing radiation has been postulated to induce multiple biological effects by direct interaction with DNA or through the formation of free radical species leading to DNA damage (Hall, 1988). These effects include gene mutations, malignant

encoding region that encodes at least one polypeptide, which encoding region is operatively linked to a transcription-terminating region.

Preferably, a radiation responsive enhancer-promoter comprises a CArG domain of an *Egr-1* promoter, a TNF-α promoter or a c-*Jun* promoter. In one preferred embodiment, an encoding region encodes a single polypeptide. A preferred polypeptide encoded by such an encoding region has the ability to inhibit the growth of a cell and, particularly a tumor cell.

An exemplary and preferred polypeptide is a cytokine, a dominant negative, a tumor suppressing factor, an angiogenesis inhibitor or a monocyte chemoattractant. More particularly, such a preferred polypeptide is TNF-α, interleukin-4, JE, ricin, PF4 Pseudomonas toxin, p53, the retinoblastoma gene product or the Wilms' tumor gene product.

Another preferred polypeptide encoded by such an encoding region has radioprotective activity toward normal tissue. An exemplary and preferred such polypeptide having radioprotective activity is interleukin-1; TNF; a tissue growth factor such as a hematopoietic growth factor, a hepatocyte growth factor, a kidney growth factor, an endothelial growth factor or a vascular smooth muscle growth factor; interleukin-6; a free radical scavenger or a tissue growth factor receptor.

Preferably, 1) a hematopoietic growth factor is interleukin-3 or a colony stimulating factor (CSF) such as GM-CSF, G-CSF and M-CSF; 2) an endothelial growth factor is basic fibroblast growth factor (bFGF); 3) a vascular smooth muscle growth factor is platelet derived growth factor (PDGF); and 4) a free radical scavenger is manganese superoxide dismutase (MnSOD).

Yet another preferred polypeptide encoded by such an encoding region has anticoagulant, thrombolytic or thrombotic activity as exemplified by plasminogen activator, a streptokinase or a plasminogen activator inhibitor.

A further preferred polypeptide encoded by such an encoding region has the ability to catalyze the conversion of a pro-drug to a drug. Exemplary and preferred such polypeptides are herpes simplex virus thymidine kinase and a cytosine deaminase.

The present invention also contemplates a pharmaceutical composition comprising a DNA molecule of the present invention and a physiologically acceptable carrier.

In another aspect, the present invention contemplates a cell transformed or transfected with a DNA molecule of this invention or a transgenic cell derived from such a transformed or transfected cell. Preferably, a transformed or transgenic cell of the present invention is a leukocyte such as a tumor infiltrating lymphocyte or a T cell or a tumor cell.

In another aspect, the present invention contemplates a process of regulating the expression of a polypeptide comprising the steps of:

(a) operatively linking a radiation responsive enhancer-promoter to an encoding region that encodes the polypeptide, which encoding region is operatively linked to a transcription-terminating region to form a DNA molecule; and

(b) exposing the DNA molecule to an effective expression-inducing dose of ionizing radiation.

In an alternate embodiment, more than one DNA molecule is prepared. Preferably, those DNA molecules comprise:

(1) a first DNA molecule comprising a radiation responsive enhancer-promoter operatively linked to an encoding region that comprises:

(a) a first encoding sequence that encodes a DNA binding domain of a first transcription factor;

(b) a second encoding sequence that encodes an activation or repression domain of a second transcription factor;

(c) a third encoding sequence that encodes a nuclear localization signal, whereby the first, second and third encoding sequences are operatively linked in frame to each other in any order with the proviso that the third encoding sequence need be present only if the first or second encoding sequence does not encode a nuclear localization signal; and

(d) a transcription-terminating region that is operatively linked to any of the first, second or third encoding sequences such that the transcription-terminating region is located 3' to all of the first, second and third encoding sequences; and

(b) exposing the tumor to an effective expression-inducing dose of ionizing radiation.

Preferably, a radiation responsive enhancer-promoter comprises a CArG domain of an *Egr-1* promoter, a TNF-α promoter or a c-*Jun* promoter and a polypeptide is a cytokine, a dominant negative, a tumor suppressing factor or an angiogenesis inhibitor.

Delivering is preferably introducing the DNA molecule into the tumor. Where the tumor is in a subject, delivering is administering the DNA molecule into the circulatory system of the subject. In a preferred embodiment, administering comprises the steps of:

(a) providing a vehicle that contains the DNA molecule; and

(b) administering the vehicle to the subject.

A vehicle is preferably a cell transformed or transfected with the DNA molecule. An exemplary and preferred transformed or transfected cell is a leukocyte such as a tumor infiltrating lymphocyte or a T cell or a tumor cell from the tumor being treated. Alternatively, the vehicle is a virus or an antibody that immunoreacts with an antigen of the tumor.

In a preferred embodiment, exposing comprises the steps of:

a) providing a radiolabelled antibody that immunoreacts with an antigen of the tumor; and

b) delivering an effective expression inducing amount of the radiolabelled antibody to the tumor.

Alternatively, a process of inhibiting growth of a tumor comprises the steps of:

(a) delivering to the tumor a therapeutically effective amount of

(1) a first DNA molecule comprising a radiation responsive enhancer-promoter operatively linked to an encoding region that comprises:

(i) a first encoding sequence that encodes a DNA binding domain of a first transcription factor;

(ii) a second encoding sequence that encodes an activation or repression domain of a second transcription factor;

distinct target cell or tissue provides opportunities for therapeutic destruction, alteration, or inactivation of that cell or tissue.

A. DNA Molecules

In one aspect, the present invention contemplates a synthetic DNA molecule comprising a radiation responsive enhancer-promoter operatively linked to an encoding region that encodes at least one polypeptide, which encoding region is operatively linked to a transcription terminating region. As used herein, the term "synthetic" indicates that a DNA molecule of the present invention is man-made (not naturally occuring) by any means including but not limited to de novo synthesis. Preferably, that synthetic DNA molecule is isolated and purified and exists substantially free of other nucleic acids, proteins and the like.

1. Radiation responsive enhancer- promoter

A promoter is a region of a DNA molecule typically within about 100 nucleotide pairs in front of (upstream of) the point at which transcription begins (i.e., a transcription start site). That region typically contains several types of DNA sequence elements that are located in similar relative positions in different genes. As used herein, the term "promoter" includes what is referred to in the art as an upstream promoter region, a promoter region or a promoter of a generalized eukaryotic RNA Polymerase II transcription unit. Exemplary and preferred promoters are the TATA box, the CAAT box and GC-rich sequence elements.

Another type of discrete transcription regulatory sequence element is an enhancer. An enhancer provides specificity of time, location and expression level for a particular encoding region (e.g., gene). A major function of an enhancer is to increase the level of transcription of an encoding region in a cell that contains one or more transcription factors that bind to that enhancer. Unlike a promoter, an enhancer can function when located at variable distances from transcription start sites so long as a promoter is present.

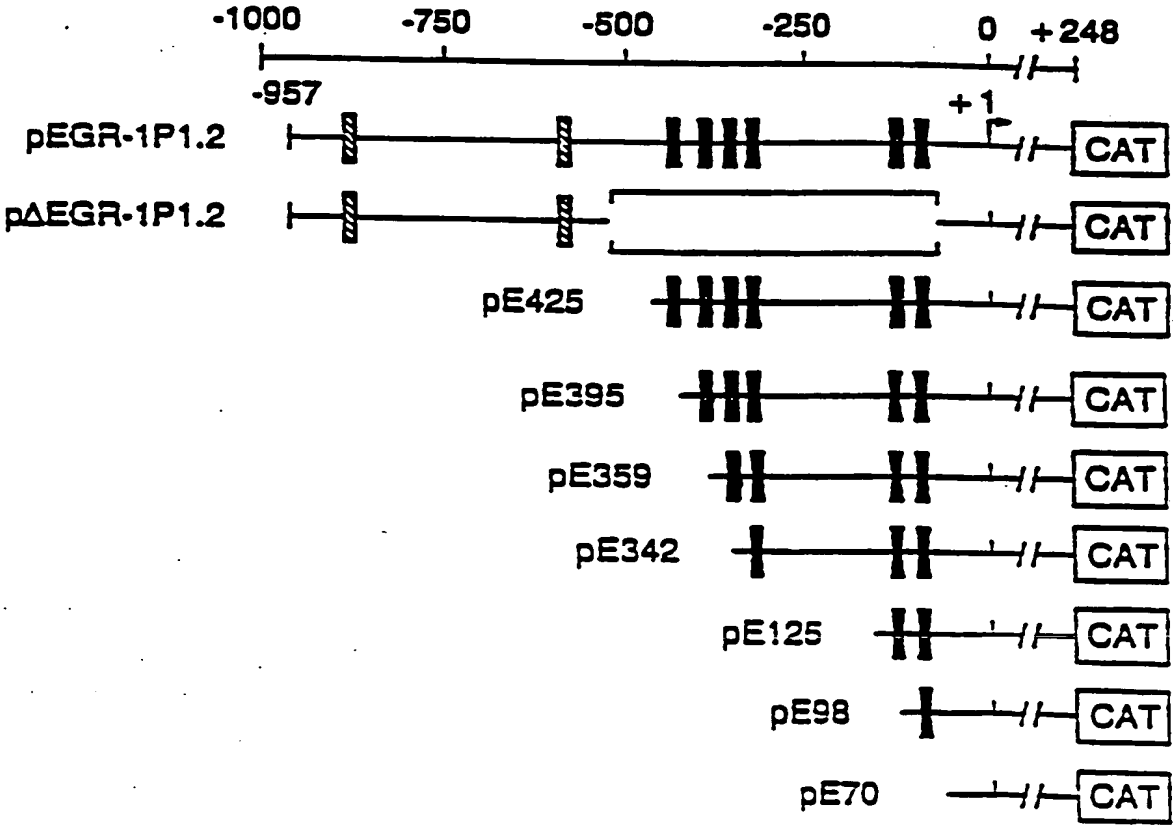
As used herein, the phrase "enhancer-promoter" means a composite unit that contains both enhancer and promoter elements. As used herein, a "radiation responsive enhancer-promoter" indicates an enhancer- promoter whose transcription controlling function is affected by ionizing radiation. Typically, upon exposure to an effective dose of ionizing radiation, a radiation responsive enhancer-promoter of the

transferase (CAT) reporter gene to form plasmid *pEgr-1* P1.2. The *Egr-1* promoter region contains several putative cis elements including six CArG domains (Christy, et al., 1989; Qureshi, et al., 1991). Treatment of *pEgr-1* P1.2 transfected cells with ionizing radiation was associated with a 4.1-fold increase in CAT activity as compared to transfected but unirradiated cells. In contrast, similar studies performed with plasmid *pΔEgr-1* P1.2 (similar to *pEgr-1* P1.2 except that nucleotides from position -550 to -50 are deleted) demonstrated little if any inducibility by x-rays. Thus, x-ray inducibility of *Egr-1* is likely mediated by sequences present between -550 and -50 of the *Egr-1* promoter.

Irradiation of cells transfected with plasmid pE425, which plasmid contains an about 491 base pair region of the *Egr-1* promoter with six CArG domains operatively linked to a CAT gene, was associated with a 3.6-fold induction of CAT activity compared to that in non-irradiated cells transfected with this construct.

A series of deleted *Egr-1* promoter constructs was next used to further define the x-ray responsive elements in pE425. Those constructs are shown schematically in Scheme 1, below.

SCHEME 1



three most distal (i.e. upstream) CArG domains. A detailed description of the radiation inducibility of the *Egr-1* gene by CArG domains upstream to the transcription initiation site can be found in Example 4 hereinafter.

Studies with the c-*fos* promoter have demonstrated that the CArG domain or serum response element is functional in inducing transcription of this gene in response to serum and other signals (Triesman, 1990). The CArG element is required for c-*fos* induction by both PKC-mediated signaling pathways and by growth factor-induced signals independent of PKC (Fisch, et al., 1987; Gilman, 1988; Buscher, et al., 1988; Sheng, et al., 1988; Stumpo, et al., 1988; Graham, et al., 1991). The kinetics of induction, as well as repression, of c-*fos* expression are similar to those of *Egr-1* in other models (Sukhatme, et al., 1988; Guis, et al., 1990). Indeed, x-ray-induced changes in c-*fos* transcripts are similar to those obtained for *Egr-1* in HL-525 cells and TPA-induced c-*fos* expression, like that for *Egr-1*, is attenuated in these cells. Studies with the c-*fos* promoter have demonstrated that the CArG domain functions as a binding site for the serum response factor (SRF) (Treisman, 1986; Prywes, et al., 1988). SRF binds, but with varying affinity, to the different CArG elements in the *Egr-1* promoter (Christy, et al., 1989).

Previous studies have demonstrated that binding of SRF to CArG in the c-*fos* promoter is not detectably altered by serum and other conditions (Treisman, 1986; Prywes, et al., 1986; Sheng, et al., 1988). Nuclear proteins from quiescent and serum-stimulated 3T3 cells have also shown little if any difference in binding to the first CArG element of the *Egr-1* promoter (Gius, et al., 1990). These findings suggest that ionizing radiation, like serum, induces a posttranscriptional modification of SRF. Other studies have demonstrated that phosphorylation of SRF is required for activation or transcription (Prywes, et al., 1988). The kinases responsible for this effect, however, remain unclear.

Alternatively, ionizing radiation may result in the modification of other proteins that interact with the SRF or CArG domain. Both SAP-1 and $p62^{TCF}$ (ternary complex factor) recognize SRF-DNA complexes (Dalton, et al., 1992; Shaw, et al., 1989), while $p62^{DBF}$ (direct binding factor) binds directly to the SRE (Ryan, et al., 1989; Walsh, 1989). Other studies have demonstrated that SRE-ZBP undergoes posttranslational modification and binds to this element (Attar, et al., 1992). One or

substantially higher at 3, 6 and 8 hours after x-ray exposure than in non-irradiated cells. Expression of the *Jun*-B and *Jun*-D gene products was also transiently increased following x-irradiation of the HL-525 cells. The kinetics of those increases in *fos* gene product expression were similar to that obtained for members of the *Jun* gene family.

The activation of *Jun* likely results in increased transcription of the AP-1 binding site following ionizing radiation exposure. The plasmid p3xTRE-CAT (containing three AP-1 sites upstream of the minimal tk promoter from plasmid pBLCAT2) was transfected into RIT-3 cells. Irradiation of p3xTRE-CAT transfectants resulted in a 3-fold increase in CAT expression.

Where RIT-3 cells transfected with a DNA molecule (c-*Jun*-CAT) comprising a 1840-base pair (-1.1 kb to +740 bp) segment of the c-*Jun* promoter placed upstream of the CAT gene were exposed to ionizing radiation, CAT expression increased about 3-fold relative to transfected, non-irradiated cells. Transfection of those cells with a plasmid having a deletion of the AP-1 site located at +150-bp (-132/+170 Δ AP-1CAT) resulted in a loss of x-ray-mediated induction of CAT expression. Thus, activated AP-1 likely participates in the transcription of c-*Jun* and the AP-1 DNA sequence is likely sufficient and necessary to confer x-ray-mediated c-*Jun* gene induction. A detailed description of x-ray induced transcription of DNA molecules containing a c-*jun* promoter can be found hereinafter in Examples 2, 3, 5 and 6.

c. TNF-α promoter

Tumor necrosis factor α (TNF-α) is a polypeptide mediator of the cellular immune response with pleiotropic activity. TNF-α acts directly on vascular endothelium to increase the adhesion of leukocytes during the inflammatory process (Bevelacqua, et al., 1989). This *in vivo* response to TNF-α was suggested to be responsible for hemorrhagic necrosis and regression of transplantable mouse and human tumors (Carswell, 1975). TNF-α also has a direct effect on human cancer cell lines *in vitro*, resulting in cell death and growth inhibition (Sugarman, et al., 1985; Old, 1985). The cytotoxic effect of TNF-α correlates with free-radical formation, DNA fragmentation, and microtubule destruction (Matthews, et al., 1988; Rubin, et al., 1988; Scanlon, et al., 1989; Yamauchi, et al., 1989; Matthews, et al.,

days, and remained elevated beyond 5 days. Furthermore, supernatant from irradiated, but not control STSAR-33 cells, was cytotoxic to TNF-α-sensitive cell line SQ-20B. A detailed description of x-ray induced transcription of DNA molecules containing the TNF-α promoter can be found hereinafter in Example 1.

2. Encoding Region

A radiation responsive enhancer-promoter is operatively linked to an encoding region that encodes at least one polypeptide. As used herein, the phrase "operatively linked" means that an enhancer-promoter is connected to an encoding region in such a way that the transcription of that encoding region is controlled and regulated by that enhancer-promoter. Means for operatively linking an enhancer-promoter to an encoding region are well known in the art. As is also well known in the art, the precise orientation and location relative to an encoding region whose transcription is controlled, is dependent inter alia upon the specific nature of the enhancer-promoter. Thus, a TATA box minimal promoter is typically located from about 25 to about 30 base pairs upstream of a transcription initiation site and an upstream promoter element is typically located from about 100 to about 200 base pairs upstream of a transcription initiation site. In contrast, an enhancer can be located downstream from the initiation site and can be at a considerable distance from that site.

a. Single polypeptide

In one embodiment, an encoding region of a DNA molecule of the present invention encodes a single polypeptide. As used herein, the term "polypeptide" means a polymer of amino acids connected by amide linkages, wherein the number of amino acid residues can range from about 5 to about one million. Preferably, a polypeptide has from about 10 to about 1000 amino acid residues and, even more preferably from about 20 to about 500 amino residues. Thus, as used herein, a polypeptide includes what is often referred to in the art as an oligopeptide (5-10 amino acid residues), a polypeptide (11-100 amino acid residues) and a protein (> 100 amino acid residues). A polypeptide encoded by an encoding region can undergo post-translational modification to form conjugates with carbohydrates, lipids, nucleic acids and the like to form glycopolypeptides (e.g., glycoproteins), lipopolypeptides (e.g. lipoproteins) and other like conjugates.

The mouse fibroblast gene is induced by PDGF. The fibroblast gene product, JE or monocyte chemoattractant protein-1 (MCP-1) is a member of a family of cytokine-like glycoproteins whose expression is induced by a mitogenic signal in monocytes, macrophages and T cells. JE has been identified, characterized and recombinantly produced from both mouse and human fibroblasts (Rollins et al., 1989). The mouse and human fibroblast gene products are designated mJE and hJE, respectively.

MCP-1 or JE is a monocyte-specific chemoattractant in vitro that is structurally related to a family of proinflammatory cytokines such as macrophage inflammatory proteins.

Exemplary and preferred polypeptides are tumor necrosis factor (TNF), interleukin-4, JE, PF4 ricin, a bacterial toxin such as Pseudomonas toxin; p53, the retinoblastoma gene product or the Wilms' tumor gene product.

In another preferred embodiment a polypeptide encoded by an encoding region has radioprotective activity toward normal cells (i.e., the polypeptide protects a normal cell or tissue from a deleterious effect of radiation). Exemplary and preferred polypeptides having radioprotective activity are interleukin-1; tumor necrosis factor; a tissue growth factor such as a hematopoietic growth factor, a hepatocyte growth factor, a kidney growth factor, an endothelial growth factor or a vascular smooth muscle growth factor; interleukin-6, a free radical scavenger or a tissue growth factor receptor.

Preferably, 1) a hematopoietic growth factor is a colony stimulating factor such as GM-CSF, G-CSF, M-CSF or interleukin-3; 2) an endothelial growth factor is basic fibroblast growth factor; 3) a vascular smooth muscle growth factor is platelet derived growth factor (PDGF); and 4) a free radical scavenger is manganese superoxide dismutase (MnSOD).

microvasculature is obliterated (Steiner, 1984). Endothelial and smooth muscle proliferation have been shown to be associated with the production of bFGF and PDGF. Clinical results may be improved by the addition of bFGF and PDGF.

In yet another preferred embodiment, the polypeptide encoded by the encoding region has anticoagulant, thrombolytic or thrombotic activity as exemplified by plasminogen activator, a streptokinase or a plasminogen activator inhibitor.

The value of coronary artery reperfusion resulting from pharmacologically induced fibrinolysis in patients with evolving myocardial infarction has been rigorously evaluated (reviewed in Tiefenbrunn, 1992; Becker, et al., 1991). Improved left ventricular function and even more impressive improvements in survival rates have been demonstrated consistently in controlled studies. Benefit is related to the restoration of myocardial blood flow. Maximal benefit is achieved with early and sustained restoration of coronary artery patency. Patients must be assessed carefully prior to initiating treatment, especially for potential bleeding hazards,and appropriate follow-up evaluation and concomitant therapy needs to be planned. However, given the overwhelming body of data now available regarding its benefits and relative safety, thrombolysis should be considered as conventional therapy for patients with acute evolving myocardial infarction.

Animal studies of stroke have been encouraging with regard to arterial recanalization and safety (reviewed in Brott, 1991; Levine, et al., 1992). Arterial recanalization has been demonstrated in patients with ischemic stroke following the administration of any one of several thrombolytic drugs. Placebo-controlled trials have not been completed, and so clinical benefit has not been established. Even though the development of brain hemorrhage has been an infrequent complication, the very high morbidity and mortality have been worrisome. Ironically, thrombolytic therapy holds promise for treatment of subarachnoid hemorrhage and perhaps also for spontaneous intracerebral hemorrhage. Human studies have been limited, but complications have been modest, and clinical outcomes have been encouraging.

In still yet another preferred embodiment, a polypeptide encoded by an encoding region has the ability to catalyze the conversion of a pro-drug to a drug or to sensitize a cell to a therapeutic agent. By way of example, cells manipulated to contain a herpes simplex virus (HSV) gene for thymidine kinase (tk) and to express

In accordance with such an embodiment, an encoding region comprises:

(a) a first encoding sequence that encodes a DNA binding domain of a first transcription factor;

(b) a second encoding sequence that encodes an activation or repression domain of a second transcription factor;

(c) a third encoding sequence that encodes a nuclear localization signal, whereby the first, second and third encoding sequences are operatively linked in frame to each other in any order with the proviso that the third encoding sequence need be present only if the first or second encoding sequence does not encode a nuclear localization signal; and

(d) a transcription-terminating region that is operatively linked to any of the first, second or third encoding sequences such that the transcription-terminating region is located 3' to all of the first, second and third encoding sequences.

As used herein, the phrase "operatively linked in frame" means that encoding sequences are connected to one another such that an open reading frame is maintained between those sequences. Means for linking DNA encoding sequences in frame are well known in the art.

DNA binding domains of transcription factors are well known in the art. Exemplary transcription factors known to contain a DNA binding domain are the GAL4, c-*fos*, c-*Jun*, lac1, trpR, CAP, TFIID, CTF, Spl, HSTF and NF-κB proteins. Preferably, a DNA binding domain is derived from the GAL4 protein.

The GAL4 protein is a transcription factor of yeast comprising 881 amino acid residues. The yeast protein GAL4 activates transcription of genes required for catabolism of galactose and melibiose. GAL4 comprises numerous discrete domains including a DNA binding domain (Marmorstein et al., 1992).

The DNA sequences recognized by GAL4 are 17 base pairs (bp) in length, and each site binds a dimer of the protein. Four such sites, similar but not identical in sequence, are found in the upstream activating sequence (UAS_G) that mediates GAL4 activation of the GAL1 and GAL10 genes, for example (Marmorstein et al., 1992).

Functions have been assigned to various parts of the 881-amino-acid GAL4 protein, including DNA binding (residues 1-65) and dimerization (residues 65-94). In

proximity to DNA in the complex, are highly conserved in these homologues. Arg 15 and Lys 20, which form phosphate salt links that anchor the first helix of the recognition module to DNA, are conserved in all but AMDR, which has His and Arg at these positions, respectively. Residue 19 is usually hydrophobic, and residue 17 is basic, except in QA-1F and LEU3. Lys 18, which makes base-specific contacts in the GAL4 complex, is conserved in all but three cases. In two of the exceptions (QA-1F and MAL63) it is Arg; in the other (PUT3), it is His. These conservations suggest that the recognition modules of these GAL4 homologues all approach DNA in a similar way (Marmorstein et al., 1992).

There are symmetrically disposed CCG sequences in known sites for LAC9, PPR1, LEU3 and PUT3. Characteristic heptad sequences suggest that several of the homologue (LAC9, QA-1F, QUTA1, PPR1) contain coiled-coil dimerization elements similar to the one in GAL4. In others such as ARGRII, HAP1, and LEU3, no obvious heptad sequences occur in the 60 residues immediately C-terminal to the recognition modules. In LEU3, the heptads lie one residue closer to the recognition module than in GAL4; in HAP1, they appear to be displaced toward the C terminus by seven residues. Some heterogeneity of dimerization structures and of linker lengths is implied by these observations (Marmorstein et al., 1992).

The closest relatives of GAL4 are LAC9, which carries out the same function in *K. lactics*, and PPR1, which regulates pyrimidine biosynthesis in *S. cerevisiae*. GAL4 and LAC9 bind to the same DNA sites; PPR1 recognizes sites with the CCG triplet separated by six, rather than 11, base pairs. GAL4 and LAC9 have similar amino-acid sequences in their linker and dimerization segments; the linker and dimerization elements of PPR1 bear no sequence similarity to those of GAL4, aside from the rough characteristics of their heptad regions (Marmorstein et al., 1992).

In a preferred embodiment, therefore, a first encoding sequence of a DNA molecule of the present invention encodes a DNA binding domain of GAL4. Preferably, that binding domain comprises amino acid residue sequences 1 to about 147 of GAL4, which numerical designations refer to amino acid residue sequences numbered consecutively beginning at the amino terminus. Thus, a first encoding sequence comprises about 444 nucleotide base pairs of the GAL4 gene, which base pairs encode amino acid residue sequences 1 to 147 of GAL4.

irradiation activates *Jun* through MAP-K modification of the A_1 domain (Binetruy, 1991 and Pulverer, 1991).

The ability of a *Jun* activation domain to stimulate transcription was demonstrated in studies of cells transformed or transfected with DNA molecules comprising such domains. HeLa and RIT-3 cells were transfected with two plasmids. Plasmid pSG-*Jun*5-253 contained the SV40 promoter (not transcriptionally responsive to radiation) upstream of an encoding region that encoded a chimeric protein (GAL4-*Jun*) comprising a sequence for Δ, A_1, and A_2 (Baichwal, 1990) and the DNA binding domain of the yeast GAL4 gene (the DNA binding domain of *Jun* was replaced with the DNA binding domain of the GAL4 gene, Baichwal, 1990). A second plasmid, G5BCAT was constructed to contain the DNA sequence that binds Gal4 protein placed 5' of the E1b TATA box and upstream of the CAT reporter gene (Baichwal, 1990).

Transcriptional activation of the activation domain of *Jun* by irradiation of transfected cells stimulated transcription and expression of the chimeric Gal-*Jun* protein, which protein bound to the Gal4 binding sequence and initiated transcription and expression of CAT. Irradiation of RIT-3 cells transfected with G5BCAT alone demonstrated no increase in CAT activity. Similar results were obtained in HeLa cells which contain the *Jun* inhibitor.

However, Hep G2 cells (which do not contain the *Jun* inhibitor; Baichwal, 1990) transfected with pSG-*Jun*5-235 and G5BCAT demonstrated no x-ray-induced activation of the Gal4-*Jun* chimeric protein. These data suggest that the Gal-*Jun* chimeric protein is activated following irradiation resulting in DNA binding to accelerate transcription of CAT.

Because X-ray induced c-*Jun* gene expression is attenuated when PKC is depleted or inhibited, the PKC inhibitor H7 was added to RIT-3 cells transfected with pSG-*Jun*5-235 and G5BCAT. H7 treatment abrogated the x-ray induced increase in CAT activity suggesting that irradiation induced PKC activation is required for gene expression (Hallahan, 1991a; Hallahan, 1991b). These data suggest that dissociation from the *Jun* inhibitor may be one mechanism of regulating radiation-mediated transcription.

residue position 515, numbered from the amino-terminus. Thus, a second encoding sequence preferably comprises nucleotide base pairs that encode amino acid residues from about residue position 414 to about residue position 515 of NF-κB (Ballard, 1992).

c. Nuclear localization signal

At least one of the encoding sequences contains a nuclear localization signal. Such a signal permits the encoded transcription factor to enter the nucleus and interact with DNA in the nucleus. Preferably, such a nuclear localization signal is contained in the first or second encoding sequence. Where a nuclear localization signal is not present in a first or second encoding sequence such a signal is contained in a third encoding sequence.

Nuclear localization signals are well known in the art. An exemplary and preferred such signal is derived from Simian Virus 40 (SV40) large T antigen. In a preferred embodiment, a SV40 nuclear localization signal comprises an amino acid residue sequence of from about 7 to about 15 amino acid residues around a lysine (Lys) residue at position 128 of SV40 large T antigen (Kalderon et al. 1984). In a more preferred embodiment a nuclear localization signal comprises the amino acid residue sequence of SV40 extending from about residue position 126 to about residue position 132.

d. Transcription-terminating region

RNA polymerase transcribes an encoding DNA sequence through a site where polyadenylation occurs. Typically, DNA sequences located a few hundred base pairs downstream of the polyadenylation site serve to terminate transcription. Those DNA sequences are referred to herein as transcription-termination regions. Those regions are required for efficient polyadenylation of transcribed messenger RNA (mRNA).

Transcription-terminating regions are well known in the art. A preferred transcription-terminating region used in a DNA molecule of the present invention comprises nucleotides 1533 to about 2157 of the human growth hormone (Seeburg, 1982).

3. Preparation of a DNA Molecule

A DNA molecule of the present invention is prepared in accordance with standard techniques well known to a skilled worker in the art. First, DNA fragments

then isolated, identified and purified using standard techniques well known in the art. If needed, an encoding DNA sequence can be amplified prior to isolation. A preferred means of amplifying a DNA sequence of interest is the polymerase chain reaction.

A wide variety and number of DNA molecules comprising a radiation responsive enhancer-promoter and an encoding region have been prepared (See Examples 1-6, hereinafter). Table 2, below, summarizes the composition of exemplary such DNA molecules.

Table 2

Plasmid Designation	Enhancer-Promoter	Encoding Region
pE425-TNF	CArG domain of *Egr-1*	TNF
pE425-CAT	CArG domain of *Egr-1*	CAT
pE425-p53	CArG domain of *Egr-1*	p53
pE425-raf 301-1	CArG domain of *Egr-1*	raf 301-1
pE425-MnSOD	CArG domain of *Egr-1*	MnSOD
pE425-Gal4/VP16	CArG domain of *Egr-1*	Gal4/VP16
c-*Jun*-CAT	c-*Jun* promoter	CAT
AP-1CAT	AP-1	CAT

a. pE425-TNF

Plasmid pE425-TNF comprises nucleotide bases from nucleotide position -425 to nucleotide position +65 (relative to the transcription start site) of the *Egr-1* gene operatively linked to an encoding region that encodes TNF-α. pE425-TNF was constructed from plasmids pE-TNF, which contains TNF cDNA, and plasmid pE425-CAT, which contains the *Egr-1* segment, a transcription-terminating region and a polyadenylation segment from CAT.

pE-TNF was digested with the restriction enzyme Pst I to yield a 1.1 kilobase (kb) fragment containing TNF cDNA. pE425-CAT was digested with the restriction enzyme Hind III to yield a 3.3 kb fragment containing the CAT gene and a 3.2 kb segment containing the *Egr-1* fragment and the polyadenylation signal from CAT. The 1.1 kb fragment from pE-TNF and the 3.2 kb fragment from pE425-CAT were

*fragment extending from nucleotide base position -425 to nucleotide base position +65.

Plasmid pCAT3m was obtained from Dr. Laimonis A. Laimins, Howard Hughes Medical Institute Research Laboratories, University of Chicago, Chicago, IL). Plasmid pE-TNF was prepared in accordance with the procedure of Wong (Wong, 1985).

b. pE425-p53

Plasmid pE425-p53 comprises an about 491 base pair fragment of the *Egr-1* promoter operatively linked to an encoding region for the tumor suppressing factor p53. pE425-p53 was constructed from a plasmid (pC53SN3; Diller, 1990) that contains p53 cDNA, and plasmid pE425-CAT, which contains the *Egr-1* segment and a transcription-terminating region, the polyadenylation segment from CAT. Plasmid pE425-CAT was prepared as described above.

c. pE425-raf 301-1

Plasmid pE425-raf 301-1 comprises an about 491 base pair fragment of the *Egr-1* promoter operatively linked to an encoding region for a serine/threonine-specific protein kinase product of an oncogene from a 3611 murine sarcoma cell. pE425-raf 301-1 was constructed from plasmids pMN301-1, which contains the raf dominant negative (Kolch, 1991), and pE425-CAT, which contains the *Egr-1* segment and a transcription-terminating region, the polyadenylation segment from CAT.

d. pE425-MnSOD

Plasmid pE425-MnSOD comprises an about 491 base pair fragment of the *Egr-1* promoter operatively linked to an encoding region for the free-radical scavenger manganese superoxide dismutase (MnSOD). pE425-MnSOD was constructed from a plasmid nMnSOD #0664 (Genentech) (Wong, 1989) which contains MnSOD cDNA and pE425-CAT, which contains the *Egr-1* segment and a transcription-terminating region, the polyadenylation segment from CAT.

e. G5-TNF

Plasmid G5-TNF comprises the DNA binding domain of the yeast GAL4 gene and the E1b minimal promoter TATA box operatively linked to an encoding region that encodes TNF-α. pG5-TNF was constructed from plasmid G5BCAT and plasmid pE-TNF.

A therapeutically effective amount of a DNA molecule that is combined with a carrier to produce a single dosage form varies depending upon the host treated and the particular mode of administration.

As is well known in the art, a specific dose level for any particular patient depends upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, rate of excretion, drug combination, and the severity of the particular disease undergoing therapy.

A composition of the present invention is typically administered orally or parenterally in dosage unit formulations containing standard, well known nontoxic physiologically acceptable carriers, adjuvants, and vehicles as desired. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intraarterial injection, or infusion techniques.

Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions are formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation can also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol.

Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

A DNA molecule of the present invention can also be complexed with a poly(L-Lysine)(PLL)-protein conjugate such as a transferrin-PLL conjugate or an asialoorosomucoid-PLL conjugate.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, syrups, solutions, suspensions, and elixirs containing inert diluents commonly used in the art, such as water. Such compositions can also comprise adjuvants, such as wetting agents, emulsifying and suspending agents, and sweetening, flavoring, and perfuming agents.

these pores or as a consequence of the redistribution of membrane components that accompanies closure of the pores. Electroporation can be extremely efficient and can be used both for transient expression of clones genes and for establishment of cell lines that carry integrated copies of the gene of interest. Electroporation, in contrast to calcium phosphate-mediated transfection and protoplast fusion, frequently gives rise to cell lines that carry one, or at most a few, integrated copies of the foreign DNA.

Liposome transformation involves encapsulation of DNA and RNA within liposomes, followed by fusion of the liposomes with the cell membrane. In addition, DNA that is coated with a synthetic cationic lipid can be introduced into cells by fusion.

Direct microinjection of a DNA molecule into nuclei has the advantage of not exposing DNA to cellular compartments such as low-pH endosomes. Microinjection is therefore used primarily as a method to establish lines of cells that carry integrated copies of the DNA of interest.

D. Process of Regulating Expression

In another aspect, the present invention contemplates a process of regulating the expression of a polypeptide. Polypeptide expression is regulated by stimulating or inhibiting transcription of an encoding region that encodes that polypeptide. In accordance with one embodiment, a process of regulating polypeptide expression comprises the steps of:

(a) operatively linking a radiation responsive enhancer-promoter to an encoding region that encodes that polypeptide, which encoding region is operatively linked to a transcription-terminating region to form a DNA molecule; and

(b) exposing the DNA molecule to an effective expression-inducing dose of ionizing radiation.

A DNA molecule used with such a method is a DNA molecule of the present invention as set forth above.

As used herein, the phrase "effective expression-inducing dose of ionizing radiation" means that dose of ionizing radiation needed to stimulate or turn on a radiation responsive enhancer-promoter of the present invention. The amount of ionizing radiation needed in a given cell depends inter alia upon the nature of that

cells transfected with plasmid pE425/250TK (containing the four distal *Egr-1* CArG domains) were responsive to x-ray treatment. The region of the *Egr-1* promoter extending from nucleotide positions -395 to -250, which region does not include the first CArG domain, was also functional in conferring x-ray inducibility to the heterologous promoter, but to a lesser extent than pE425/250TK. X-ray inducibility of CAT expression was also observed in cells transfected with a plasmid comprising only one CArG domain.

By way of further example, TNF-α protein expression was induced by ionizing radiation in cells transfected with plasmid pE425-TNF. SQ-20B, RIT-3 and HL-525 cells were transfected with plasmid pE425-TNF by DEAE precipitation. Transfected cells were exposed to 10 Gy of x-radiation at a rate of 1 Gy/minute. TNF-α expression was increased about 2-fold, 5-fold and 4-fold, respectively in SQ-20B, RIT-3 and HL-525 cells when compared to transfected, non-irradiated cells.

By way of still further example, CAT expression was induced by ionizing radiation in RIT-3 cells transfected with plasmid c-*Jun*-CAT, which plasmid comprises a 1100 base pair segment of the c-*Jun* promoter operatively linked to a CAT gene. Cells were cotransfected with an SV40 promoter-β galactosidase expression vector to control for transfection efficiency.

Transfectants were irradiated (10 Gy, 1 Gy/min, GE Maxitron) 40 hours after transfection. CAT was extracted 6 hours after irradiation. CAT activity increased about 3-fold following irradiation of RIT-3 cells transfected with pc-*Jun*-CAT. β gal expression was not affected by radiation. Ionizing radiation did not increase CAT expression in cells transfected with a plasmid comprising the minimal *Jun* promoter (nucleotide base position-18 to nucleotide base position +170 relative to the transcription start site) operatively linked to CAT.

The data set forth above show that ionizing radiation can be used as a trigger to regulate transcription of an encoding region in a DNA molecule of the present invention and expression of a polypeptide encoded by that region.

In an alternate embodiment, polypeptide expression is regulated by the use of two DNA molecules. One of those DNA molecules comprises a radiation responsive enhancer-promoter operatively linked to an encoding region that comprises:

Transfected cells were irradiated with 10 Gy of x-rays. CAT activity increased in the irradiated, transfected HeLa and RIT-3 cells as compared to transfected, non-irradiated cells.

By way of further example, irradiation induced an increase in TNF-α expression in RIT-3 cells transfected with plasmids pE425-Gal4/VP-16 and pG5-TNF. Plasmid pE425-Gal4/VP-16 comprises an about 491 base pair fragment of the *Egr-1* promoter containing 6 CArG domains, which fragment is operatively linked to an encoding region comprising a first encoding sequence encoding DNA binding domain of Gal4 operatively linked in frame to a second encoding sequence encoding the activation domain of viral protein VP-16. Plasmid G5-TNF comprises a DNA segment that binds the Gal4 binding domain operatively linked to minimal promoter operatively linked to an encoding region that encodes TNF-α. RIT-3 cells were cotransfected with the pE425-Gal/VP16 and G5-TNF plasmids using lipofaction. Transfected cells were irradiated 36 hours following transfection and TNF was assayed 10 hours following irradiation. The concentration of intracellular TNF increased about 9-fold as compared to cells transfected with G5-TNF alone.

Where regulating is inhibiting, an encoding region preferably comprises:

(a) a first encoding sequence that encodes a DNA binding domain of a first transcription factor;

(b) a second encoding sequence that encodes an activation or repression domain of a second transcription factor;

(c) a third encoding sequence that encodes a nuclear localization signal, whereby the first, second and third encoding sequences are operatively linked in frame to each other in any order with the proviso that the third encoding sequence need be present only if the first or second encoding sequence does not encode a nuclear localization signal; and

(d) a transcription-terminating region that is operatively linked to any of the first, second or third encoding sequences such that the transcription-terminating region is located 3' to all of the first, second and third encoding sequences.

Preferably the second encoding sequence encodes the repression domain of the Wilms' tumor suppressor gene WT1 or the repression domain of *Egr-1*. A radiation

provide a rationale for the use of lymphokine gene-transfected tumor cells that are activated by irradiation as a modality for cancer therapy.

Ricin is a cytotoxin that inactivates mammalian ribosomes by catalyzing the cleavage of the N-glycosidic bond of 28S rRNA (Endo & Tsurngi, 1987). This enzyme is extremely toxic when given systemically, but may be localized to tumor through the use of radiation targeting of the gene encoding ricin.

The transforming growth factor type alpha gene has been fused to modified Pseudomonas toxin gene from which the cell-recognition domain has been deleted (Chaudhary, et al., 1987). The chimeric gene has been expressed in Escherichia coli, and the chimeric protein, PE40-TGF-alpha, has been highly purified. PE40-TGF-alpha kills cells expressing epidermal growth factor receptors and has little activity against cells with few receptors. This chimeric protein might be useful in treating cancers that contain high numbers of epidermal growth factor receptors. The gene encoding pseudomonas toxin or its chimeric may be targeted by radiation to eliminate the potential systemic sequelae of this toxin.

Delivering is preferably injecting the DNA molecule into the tumor. Where the tumor is in a subject delivering is preferably administering the DNA molecule into the circulatory system of the subject. In a more preferred embodiment, administering comprises the steps of:

(a) providing a vehicle that contains the DNA molecule; and

(b) administering the vehicle to the subject.

A vehicle is preferably a cell transformed or transfected with the DNA molecule or a transfected cell derived from such a transformed or transfected cell. An exemplary and preferred transformed or transfected cell is a leukocyte such as a tumor infiltrating lymphocyte or a T cell or a tumor cell from the tumor being treated. Means for transforming or transfecting a cell with a DNA molecule of the present invention are set forth above.

Human lymphocytes can also be transfected with radiation-inducible plasmid constructs using existing technology including retroviral mediated gene transfer (Overell, et al., 1991; Fauser, 1991). In an exemplary embodiment, LAK cells which tend to home in on the tumor site in question with some degree of preference though as is well known, they will also distribute themselves in the body in other

gene to dividing tumor cells in the nervous system, where most endogenous cells are not dividing. Radiation will be used to enhance the specificity of delivery or activation of transcription of the tk gene only in irradiated areas.

Antibodies have been used to target and deliver DNA molecules. An N-terminal modified poly(L-lysine) (NPLL)-antibody conjugate readily forms a complex with plasmid DNA (Trubetskoy et al., 1992). A complex of monoclonal antibodies against a cell surface thrombomodulin conjugated with NPLL was used to target a foreign plasmid DNA to an antigen-expressing mouse lung endothelial cell line and mouse lung. Those targeted endothelial cells expressed the product encoded by that foreign DNA.

In a preferred embodiment exposing comprises the steps of:

a) providing a radiolabelled antibody that immunoreacts with an antigen of the tumor; and

b) delivering an effective expression inducing of the radiolabelled antibody to the tumor.

The effecacy of using antibodies to target radiotherapy has been demonstrated including the modeling of dose to tumor and normal tissue from intraperitoneal radioimmunotherapy with alpha and beta emitters[4]. This technology has been applied to *in vivo* experiments. Astatine-211 labeling of an antimelanoma antibody and its Fab fragment using N-succinimidyl p-astatobenzoate: comparison in vivo with the p-(125)iodobenzoyl conjugate[5].

Alternatively, a process of inhibiting growth of a tumor comprises the steps of:

a) delivering to the tumor a therapeutically effective amount of

(1) a first DNA molecule comprising a radiation responsive enhancer-promoter operatively linked to an encoding region that comprises

(i) a first encoding sequence that encodes a DNA binding domain of a first transcription factor;

(ii) a second encoding sequence that encodes an activation or repression domain of a second transcription factor;

(iii) a third encoding sequence that encodes a nuclear localization signal, whereby the first, second and third encoding sequences are

mRNA 3 hours after irradiation in cell line STSAR-13 and at 6 hours in cell line STSAR-48. These data show that TNF-α gene expression is increased after radiation.

Figure 1 exhibits the influence of TNF-α on radiation lethality of TNF-α-producing human sarcomas and TNF-α-nonproducing human tumor cells. The solid lines indicate the effects of radiation alone, and the dashed lines indicate the effects of both TNF-α and irradiation. Representative survival data for cell line STSAR-33 are shown in the graph to the left, A. The lower dashed line represents survival of cells with TNF-α at 1000 units/ml, corrected for a plating efficiency (PE) of 30%. The survival of human epithelial tumor cells (SQ-20B) irradiated with TNF-α (10 units/ml and 1000 units/ml) is shown in the middle graph, B. Survival data for SQ-20B show an additive effect of TNF-α (1000 units/ml). Survivals with TNF-α are corrected for 85% killing with TNF-α alone. Radiation survival data for HNSCC-68 is shown in the graph to the right, C. A nonlethal dose of TNF-α (10 units/ml) was added 24 hr before irradiation.

As can be seen from these results and from information discussed in Example 1, the tumor necrosis factor α is increased after treatment with x-rays. Both mRNA and TNF-α proteins were increased.

Although DNA-damaging agents other than ionizing radiation have been observed to induce expression of variety of prokaryotic and mammalian genes, the TNF-α gene is the first mammalian gene found to have increased expression after exposure to ionizing radiation. This gene is not categorized as a DNA repair gene.

A DNA molecule of the present invention has uses other than inhibition of tumor growth. Exemplary such uses are summarized below in Table 3.

Table 3 (contd)

Use	Encoded Polypeptide	Application to Disease
Correct Deficiencies Leading to Neurodegenerative Disease	Nerve Growth Factor	Alzheimer's Disease
Provide Treatment Component for Diabetes	Insulin	Diabetes
Disease of DNA Repair Abnormalities	ERCC-1, XRCC-1	Ataxia Telangiectasia Xeroderma Pigmentosum

The following examples illustrate particular embodiments of the present invention and are not limiting of the specification and claims in any way.

EXAMPLE 1: Increased Tumor Necrosis Factor α mRNA After Cellular Exposure to Ionizing Radiation

A. Protein Products

To investigate TNF-α protein production after x-irradiation, the levels of TNF-α in the medium of human tumor cell lines and fibroblasts were quantified by the ELISA technique (Sariban, et al., 1988) before and after exposure to 500-cGy x-rays. Five of 13 human bone and soft tissue sarcoma cell lines (STSAR-5, -13, -33, -43, and -48) released TNF-α into the medium after irradiation, whereas TNF-α levels were not elevated in supernatant from normal human fibroblast cell lines (GM-1522 and NHF-235) and four human epithelial tumor cell lines (HN-SCC-68, SCC-61, SCC-25, and SQ-20B) after exposure to radiation. The assay accurately measures TNF-α levels between 0.1 and 2.0 units per ml (2.3×10^6 units/mg) (Saribon, et al., 1988). Tumor cell line STSAR-13 produced undetectable amounts of TNF-α before x-irradiation and 0.35 units/ml after x-ray exposure. Cell lines STSAR-5 and -33 responded to x-irradiation with increases in TNF-α concentrations of >5- to 10-fold; however quantities above 2 units/ml exceeded the range of the assay (Saribon, et al.,

cytotoxic to four of five TNF-α-producing sarcomas (STSAR-5, -13, -33, and -43). The plating efficiency (PE) was reduced by 60-90% at 1000 units/ml in these lines. Radiation-survival analysis of cell line STSAR-33 was performed with TNF-α (10 units/ml). The radiosensitivity (D_0), defined as the reciprocal of the terminal slope of the survival curves was 80.4 cGy for cell line STSAR-33. When TNF-α was added 20 hr before irradiation, the D_0 was 60.4 cGy. Surviving fractions were corrected for the reduced PE with TNF-α. Thus, the interaction between TNF-α and radiation in STSAR-33 cells was synergistic (Dewey, 1979).

Sublethal concentrations of TNF-α (10 units/ml) enhanced killing by radiation in cell line STSAR-33, suggesting a radiosensitizing effect of TNF-α. The surviving fraction of cell line STSAR-5 at 100-700 cGy was lower than expected by the independent killing of TNF-α and x-rays, although the D_0 values were similar. Thus, the interaction between TNF-α and radiation is additive (Dewey, 1979) in STSAR-5 cells. Cell lines STSAR-13 and STSAR-43 were independently killed with x-rays and TNF-α, and no interaction was observed.

To determine the possible interactions between TNF-α and x-rays in non-TNF-α producing cells, human epithelial tumor cells (SQ-20B and HNSCC-68) were irradiated 20 hr after TNF-α was added. These cell lines do not produce TNF-α in response to ionizing radiation. TNF-α (1000 units/ml) was cytotoxic to SQ-20B and SCC-61 cells, reducing the PE by 60-80%. The D_0 for cell line SQ-20B is 239 cGy. With TNF-α (1000 units/ml) added 24 hr before x-rays, the D_0 was 130.4 cGy. Therefore, a synergistic interaction (Dewey, 1979) between TNF-α and x-rays was demonstrated in this cell line. TNF-α added after irradiation did not enhance cell killing by radiation in cell lines SQ-20B. Nonlethal concentrations of TNF-α (10 units/ml) resulted in enhanced radiation killing in cell line HNSCC-68, providing evidence that TNF-α may sensitize some epithelial as well as mesenchymal tumor cell lines to radiation.

The following specific methods were used in Example 1.

Cell Lines. Methods of establishment of human sarcoma and epithelial cell lines have been described (Weichselbaum, et al., 1986; 1988). Culture medium for epithelial tumor cells was 72.5% Dulbecco's modified Eagle's medium/22.5% Ham's nutrient mixture F-12 (DMEM/F-12 (3:1))5% fetal bovine serum (FBS), transferrin

EXAMPLE 2: Increased c-*Jun* Expression After Exposure to Ionizing Radiation

Another embodiment of a DNA molecule derives from the c-*Jun* protooncogene and related genes. Ionizing radiation regulates expression of the c-*Jun* protooncogene, and also of related genes c-*fos* and *Jun*-B. The protein product of c-*Jun* contains a DNA binding region that is shared by members of a family of transcription factors. Expression level after radiation is dose dependent. The c-*Jun* gene encodes a component of the AP-1 protein complex and is important in early signaling events involved in various cellular functions. AP-1, the product of the protooncogene c-*Jun* recognizes and binds to specific DNA sequences and stimulates transcription of genes responsive to certain growth factors and phorbol esters (Bohmann, et al., 1987; Angel, et al., 1988). The product of the c-*Jun* protooncogene contains a highly conserved DNA binding domain shared by a family of mammalian transcription factors including *Jun*-B, *Jun*-D, c-*fos*, *fos*-B, *fra*-1 and the yeast GCN4 protein.

In addition to regulating expression of the c-*Jun* gene, c-*Jun* transcripts are degraded posttranscriptionally by a labile protein in irradiated cells. Posttranscriptional regulation of the gene's expression is described in Sherman, et al., 1990.

Contrary to what would be expected based on previous DNA damage and killing rates for other agents, decreasing the dose rate, for example, from 14.3 Gy/min to 0.67 Gy/min. was associated with increased induction of c-*Jun* transcripts.

Maximum c-*Jun* mRNA levels were detectable after 50 Gy of ionizing radiation. Similar kinetics of c-*Jun* induction were observed in irradiated human U-937 monocytic leukemia cells and in normal human AG-1522 diploid fibroblasts. Treatment of AG-1522 cells with ionizing radiation was also associated with the appearance of a minor 3.2-kb c-*Jun* transcript.

The following methods were used in Example 2.

Cell Culture. Human HL-60 promyclocytic leukemia cells, U-937 monocytic leukemia cells (both from American Type Culture Collection), and AG-1522 diploid foreskin fibroblasts (National Institute of Aging Cell Repository, Camden, NJ) were grown in standard fashion. Cells were irradiated using either Philips RT 250

suggested that this effect is mediated in part by a protein kinase C (PKC) dependent pathway.

Jun homodimers and *Jun/fos* heterodimers regulate transcription by binding to *AP1* sites in certain promoter regions (Curran and Franza, 1988). The *Jun* and *fos* genes are induced following x-ray exposure in human myeloid leukemia cells suggests that nuclear signal transducers participate in the cellular response to ionizing radiation.

The *Egr-1* and *Jun* genes are rapidly and transiently expressed in the absence of de novo protein synthesis after ionizing radiation exposure. *Egr-1* and *Jun* are most likely involved in signal transduction following x-irradiation. Down-regulation of PKC by TPA and H7 is associated with attenuation of *Egr-1* and *Jun* gene induction by ionizing radiation, implicating activation of PKC and subsequent induction of the *Egr-1* and *Jun* genes as signaling events which initiate the mammalian cell phenotypic response to ionizing radiation injury.

Control RNA from unirradiated cells demonstrated low but detectable levels of *Egr-1* and *Jun* transcripts. In contrast, *Egr-1* expression increased in a dose dependent manner in irradiated cells. Levels were low but detectable after 3 Gy and increased in a dose dependent manner following 10 and 20 Gy. Twenty Gy was used in experiments examining the time course of gene expression so that transcripts were easily detectable. Cells remained viable as determined by trypan blue dye exclusion during this time course. A time dependent increase in *Egr-1* and *Jun* mRNA levels was observed. SQ-20B cells demonstrated coordinate increases in *Egr-1* and *Jun* expression by 30 minutes after irradiation that declined to baseline within 3 hours. In contrast, *Egr-1* transcript levels were increased over basal at 3 hours while *Jun* was increased at one hour and returned to basal at 3 hours in AG1522. *Jun* levels were increased at 6 hours in 293 cells while *Egr-1* was increased at 3 hours and returned to basal levels by 6 hours.

To determine whether *Egr-1* and *Jun* participated as immediate early genes after x-irradiation, the effects of protein synthesis inhibition by cycloheximide were studied in cell lines 293 and SQ-20B after x-ray exposure. Cycloheximide treatment alone resulted in a low but detectable increase in *Egr-1* and *Jun* transcripts normalized to 7S. In the absence of CHI, the level of *Egr-1* and *Jun* expression returned to

remain unclear. Recent studies have demonstrated that ionizing radiation exposure is associated with activation of certain immediate-early genes that code for transcription factors. These include members of the *Jun/fos* and early growth response (*Egr*) gene families (Sherman, et al., 1990; Hallahan, et al, 1991). Other studies have demonstrated that x-rays induce expression and DNA binding activity of the nuclear factor κB (NF-κB; Brach, et al., 1991).

The activation of these transcription factors may represent transduction of early nuclear signals to longer term changes in gene expression which constitute the response to ionizing radiation. In this context, irradiation of diverse cell types is also associated with increased expression of the TNF, PDGF, FGF and interleukin-1 genes (Hallahan, et al., 1989; Witte, et al, 1989; Woloschak, et al., 1990; Sherman, et al., 1991). Expression of cytokines is conceivably involved in the repair and repopulation associated with x-ray-induced damage to tissues, and may explain some of the organismal effects of ionizing radiation (Hall, 1988). Moreover, it is possible that immediate-early transcription factors serve to induce these changes in gene expression.

The present studies relate to mechanisms responsible for x-ray-induced activation of the *Egr-1* gene (also known as zif/268, TIS-8, NFGI-A and Krox-24; Sukhatme, et al., 1988; Christy, et al., 1988; Milbrandt, 1987; Lemaire, et al., 1988; Lim, et al., 1987). The *Egr-1* gene encodes a 533-amino acid nuclear phosphoprotein with a Cys_2-His_2 zinc finger domain that is partially homologous to the corresponding domain in the Wilms tumor-susceptibility gene (Gessler, 1990). The *Egr-1* protein binds to the DNA sequence CGCCCCCGC in a zinc-dependent manner and functions as a regulator of gene transcription (Christy, et al, 1989; Cao, et al., 1990; Gupta, et al, 1991). Both mitogenic and differentiation signals have been shown to induce the rapid and transient expression of *Egr-1* in a variety of cell types. For example, the *Egr-1* gene is induced after mitogenic stimulation of Balb/c-3T3 cells by serum, PDGF or FGF (Lau, et al, 1987; Sukhatme, et al., 1987). The *Egr-1* gene is also induced during: 1) cardiac and neuronal differentiation of the pleuripotent EC line (Sukhatme, et al., 1988); and 2) monocytic differentiating of human myeloid leukemia cell lines (Kharbanda, et al., 1991; Bernstein, et al., 1991). While *Egr-1* transcription is activated by the protein tyrosine kinase activity of v-src and v-fps

transcription start site to position +248 was ligated to the CAT reporter gene (plasmid p*Egr-1* P1.2). This region contains several putative cis elements including two AP-1 sites and six CArG domains (Christy, et al., 1989; Gius, et al., 1990). Treatment of the p*Egr-1*P1.2 transfected cells with ionizing radiation was associated with a 4.1-fold increase in CAT activity as compared to transfected but unirradiated cells. In contrast, similar studies performed with plasmid pΔ*Egr-1* P1.2 (-550 to -50 deleted) demonstrated little if any inducibility by x-rays. These data suggested that x-ray inducibility of *Egr-1* is mediated by sequences present between -550 and -50 of the *Egr-1* promoter. Indeed, irradiation of pE425 transfected cells was associated with a 3.6-fold induction of CAT activity compared to that in non-irradiated cells transfected with this construct.

A series of deleted *Egr-1* promoter constructs was next used to further define the x-ray responsive elements in pE425. These constructs have been previously described and are shown schematically in Scheme 1. Sequential deletion of the three distal CArGs progressively decreased CAT activity. pE395 (first CArG deleted) conferred x-ray inducibility to a lesser extent than pE425. Deletion of the first and second (pE359) CArGs resulted in further decreases in CAT activity, while deletion of the first three CArG domains (pE342) was associated with little if any increases in CAT activity. Taken together, these findings supported the hypothesis that the three distal CArG elements confer x-ray inducibility of the *Egr-1* gene.

Other studies were performed with fragments of the *Egr-1* promoter linked to HSV-TK and the CAT gene. There was no detectable inducibility of pTK35CAT by x-rays. In contrast, pE425/250TK, which contains the four distal CArG domains, was more responsive to x-ray treatment than pTK35CAT. The region from -395 to -250, which excludes the first CArG element, was also functional in conferring x-ray inducibility to the heterologous promoter, but to a lesser extent than pE425/250TK. While these findings provided further support for the involvement of CArG domains in x-ray induced *Egr-1* transcription, other sequences between these domains could be the functional cis elements. X-ray inducibility of pTK35CAT transcription was also demonstrated with the first CArG with seven base pairs of the 5' and 3' flanking sequences (pSRE1TK).

SSC-0.1% SDS at 60°C for 1 h. Signal intensity was determined by laser densitometry and normalized to that for the actin control.

Nuclear run-on assays. Nuclei were isolated from 10^8 cells and suspended in 100 μl glycerol buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA). An equal volume of reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 100 mM KCl, 1 mM ATP, 1 mM CTP, 1 mM GTP, and 5 mM dithiothreitol) was added to the nuclei in suspension and incubated at 26°C for 45 min with 250 μCi (α-^{32}p) UTP (3000 Ci/mmol; Dupont, Boston, MA). The nuclear RNA was isolated as described (Kharbanda, et al., 1991) and hybridized to the following DNAs: 1) a 1.1-kb BamHI insert of a human β-globin gene (negative control) (Hallahan, et al., 1991); 2) a PstI digest of the pA1 plasmid containing a fragment of the chicken β-actin gene (positive control) (Dignam, et al., 1983); and 3) the 0.7-kb insert of the murine *Egr-1* cDNA (Sukhatme, et al., 1988). The digested DNAs were run in 1% agarose gels and transferred to nitrocellulose filters. Hybridizations were performed with 10^7 cpm of ^{32}P-labeled RNA/ml in 10 mM Tris-HCl, pH 7.5, 4x SSC, 1 mM EDTA, 0.1% SDS, 2x Denhardt's solution, 40% formamide, and 100 μg/ml yeast tRNA for 72 h at 42°C. The filters were washed in: a) 2x SSC-0.1% SDS at 37°C for 30 min; b) 200 ng/ml RNase A in 2x SSC at room temperature for 5 min; and c) 0.1x SSC-0.1% SDS at 42°C for 30 min.

Reporter assays. The p*Egr-1*P1.2, pE425, pE395, pE359, pE342, pE125, pE98 and pE70 constructs were prepared as described (26). pE425/250TK was constructed by cloning a HindIII-SmaI fragment from pE425, spanning the region -425 to -250 of the *Egr-1* promoter, upstream of the herpes simplex virus thymidine kinase (HSV-TK) promoter in plasmid pTK35CAT (Homma, et al., 1986). pE395/250TK was constructed in the same manner using a HindIII-SmaI fragment from pE395. pSRE1TK contains the 5'-most distal or first CArG domain in the *Egr-1* promoter along with seven base pairs of the 5' and 3' flanking sequences cloned into the SalI-BamHI site of pTK35CAT (Homma, et al., 1986). The constructs were transfected into cells using the DEAE-dextran technique (Treisman, et al., 1990). Cells ($2x10^7$) were incubated in 1 ml of Tris-buffered saline solution (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, and 0.7 mM $MgCl_2$ containing 0.4 mg DEAE-dextran and 8 μg plasmid, at 37°C for 45 min. The cells

suggests that ionizing radiation induces the *Egr-1* and TNF genes by distinct signaling pathways in these cells.

These studies further demonstrate that x-ray-induced *Egr-1* expression is regulated at least in part by transcriptional mechanisms. Nuclear run-on assays demonstrated an increase in the rate of *Egr-1* gene transcription following ionizing radiation. Moreover, analysis of the full length *Egr-1* promoter (p*Egr-1*P1.2) in transient expression assays demonstrated inducibility by ionizing radiation. Transfection of pΔ*Egr-1*P1.2 (-550 to -50 deleted) and pE425 provided additional evidence that the promoter region containing the six CArG elements was responsible for conferring x-ray inducibility. These findings were supported by the use of several other deleted promoter constructs which indicated that the region encompassing the first three CArG elements is functional in the x-ray response.

In this context, sequential deletion of these distal CArGs progressively eliminated the x-ray response. The four distal CArGs also conferred x-ray inducibility to a heterologous promoter and this effect was decreased by deleting the first CArG domain. More importantly, studies with the first CArG domain demonstrated that this element was sufficient to confer the x-ray response. Taken together, these findings strongly support the CArG domain as the radiation responsive element.

EXAMPLE 5: Radiation Signalling Mediated by *Jun* Activation

Ionizing radiation produces a wide range of effects on cells which include induction of mutations, lethality, malignant transformation in some surviving cells, cell cycle arrest, and subsequent proliferation of cells. *Jun*, a transcription factor that is central to tumor promotion, proliferation and cell cycle regulation, is activated by DNA damaging agents in mammalian cells (Devary, 1991 and Bernstein, 1989). One proposed mechanism of *Jun* activation is through dissociation of *Jun* from an inhibitor of *Jun* transcription (Baichwal, 1990).

To investigate protein binding to the AP-1 sequence following irradiation, nuclear proteins were extractd from irradiated human sarcoma cell line RIT-3 cells at 5 minutes intervals for 30 minutes following exposure to 10 Gy. The AP-1, NFκB, SP-1 and CTF binding sequences labeled with ^{32}P were incubated with cell extracts. DNA-protein mixtures were then separated by electrophoresis. An increase in nuclear protein binding to AP-1 DNA sequences was found at 10 to 20 minutes

Transfection of the -1.1kb to +740-bp region of the c-*Jun* promoter (c-*Jun*-CAT) demonstrated a 3-fold increase in gene expression following exposure to ionizing radiation. Transfection of the plasmid with a deletion of the AP-1 site located at +150-bp (-132/+170 Δ AP-1CAT) resulted in a loss of x-ray-mediated induction. These results suggest that activated AP-1 participates in the transcription of c-*Jun* and that the AP-1 DNA sequence is sufficient and necessary to confer x-ray-mediated gene induction.

Several regulatory and DNA binding domains exist within the *Jun* protein. Close to the DNA binding domain is a region designated as A_2, which is required to activate transcription (reviewed in (Lewin, 1991). A_1, an additional transcriptional activation domain is found near the N terminus adjacent to a region termed Delta (Δ) which is proposed to bind a cellular protein that inhibits the transcriptional activating properties of *Jun* (Baichwal, 1990 and Baichwal, 1991). *Jun* transcriptional activity can be conferred through either or both activation domains A_1 and A_2. Phorbol ester treatment results in the modification of the *Jun* protein by a protein kinase C (PKC)-dependent phosphorylation of the A_1 region and thereby autoinduces transcription of c-*Jun* (Binetruy, 1991 and (Pulverer, 1991).

Increased *Jun* binding to AP-1 sequences following irradiation indicate that *Jun* protein is modified following irradiation. Taken together with the recent findings that PKC is activated following irradiation of cells and that PKC depletion suppress c-*Jun* induction by irradiation (Hallahan, 1992), the data suggest that X-ray exposure activates *Jun* through PKC modification of the A_1 domain (Binetruy, 1991 and Pulverer, 1991).

Two plasmids were transfected into HeLa and RIT-3 cells to study activation of the transcriptional potential of *Jun* protein following irradiation. pSG-*Jun*5-253 contains the SV40 promoter, which is not transcriptionally responsive to radiation, upstream of the coding sequence for Δ, A_1, and A_2 (Baichwal, 1990). The DNA binding domain of *Jun* was replaced with the DNA binding domain of the yeast GAL4 gene which encodes a protein involved in yeast transcriptional regulation (Baichwal, 1990). A second plasmid, G5BCAT contains the DNA sequence which binds Gal4 protein placed 5' of the E1b TATA box upstream of the CAT reporter gene (Baichwal, 1990). When the activation domain of *Jun* protein becomes

RIT-3 cells cotransfected with CMV-*Jun*, pSG*Jun*5-235 and G5BCAT do not demonstrate an increase in transcription as compared to these transfectants treated with identical conditions, but no irradiation. These data suggest that dissociation from the *Jun* inhibitor may be one mechanism of regulating radiation-mediated transcription.

The following methods were used in Example 5.

Nuclear Extracts. Nuclear extracts were prepared according to previously described methods (Schreiber, 1989) at 10,20,30, and 60 min. after irradiation. RIT-3 cells (10^6) were washed in 10 ml PBS, scraped, and pelleted by centrifugation at 1500 g for 5 min. The pellet was resuspended in 1 ml PBS, transferred into an Eppendorf tube and pelleted again for 15 sec. PBS was removed and the cell pellet resuspended in 400 μl of cold buffer A (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 1 mM DTT; 0.5 mM PMSF). The cells were allowed to swell on ice for 15 min, followed by the addition of 25 ml of 10% NP-40. The mixture was centrifuged for 30 sec and the nuclear pellet resuspended in 50 μl ice-cold buffer B (20 mM HEPES pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF) for 15 min and the nuclear extract centrifuged for 5 min. Protein content was determined by the Bradford method (Bio-Rad). The AP-1 consensous sequence DNA (BRL-GIBCO) was end-labeled with (^{32}P)dATP using DNA polymerase I Klenow fragment.

Binding Assays. Binding assays were performed by incubating the end-labeled DNA (1 ng) with 10 μg nuclear protein, 75 mM KCl and 1 μg/ml poly (dI-dC) in a 20 μl reaction for 20 min at room temperature.

Competition Assays. Competition studies were performed using oligonucleotides corresponding to known cis-acting elements AP-1, and Oct-1 (BRL-GIBCO) at a 100-fold molar excess as compared to the labeled fragments. The reaction products were separated by 5% polyacrylamide gel electrophoresis, dried and analyzed by autoradiography.

Antisera Studies. Antisera to *Jun* and *fos* proteins reduced radiation-induced AP-1 binding. A. Antiserum to human transcription factors c-*Jun* (amino acids 73-87, Ab-2, Oncogene Sci.), c-*Jun*, *Jun*-B, *Jun*-D (Cambridge Research, log OA-11-837), and c-*fos* (amino acids 4-17, Ab-2, Oncogene Sci and (amino acids 1-

of cells to x-rays is associated with activation of the c-*Jun*/c-*fos* and *Egr-1* gene families which code for transcription factors. Other studies have demonstrated that ionizing radiation induces expression and DNA binding activity of the nuclear factor κB (NF-κB). The activation of transcription factors likely represents a critical control point in transducing early nuclear signals to longer term changes in gene expression that reflect the response to x-ray-induced damage. Studies have demonstrated that radiation treatment is associated with increased expression of certain cytokines, including TNF, platelet-derived growth factor, fibroblast growth factor and interleukin-1. The increase in TNF expression following exposure to ionizing radiation is regulated by transcriptional mechanisms, although it is not known which DNA binding proteins confer this inducibility.

The c-*Jun* gene codes for the major form of the 40-44 kD AP-1 transcription factor. As observed in irradiated cells, this gene is induced as an immediate early event in response to phorbol esters and certain growth factors. The *Jun*/AP-1 complex binds to the heptomeric DNA consensus sequence TGA$^{G}/_{C}$TCA. The DNA binding domain of c-*Jun* is shared by a family of transcription factors, including *Jun*-B, *Jun*-D and c-*fos*. Moreover, the affinity of c-*Jun* binding to DNA is related to the formation of homodimers or heterodimers with products of the *fos* gene family.

Jun-B also forms dimers and binds to the AP-1 element, although the trans-acting properties of *Jun*-B differ from those of c-*Jun*. While the product of the *Jun*-D gene also interacts with c-*fos* and has similar binding properties to that of c-*Jun*, the function of *Jun*-D is unknown. Certain insights are available regarding the signals which contribute to the regulation of these genes. For example, the finding that phorbol esters activate c-*Jun* transcription in diverse cell types has implicated the involvement of a phosphorylation- dependent mechanism. A similar pathway appears to play a role, at least in part, in the induction of c-*Jun* expression by ionizing radiation. In this regard, prolonged treatment with phorbol esters to down-regulate PKC is associated with decreases in the effects of x-rays on c-*Jun* transcription. Furthermore, non-specific inhibitors of PKC, such as the isoquinolinesulfonamide derivative H7, block x-ray-induced c-*Jun* expression. Taken together with the demonstration that ionizing radiation induces an activity with characteristics of PKC,

were also studied. c-*fos* transcripts were present at low levels in HL-205 cells and there was little if any effect of ionizing radiation on expression of this gene. Similar findings were obtained for *fos*-B. In contrast, while expression of c-*fos* and *fos*-B was also low in HL-525 cells, x-ray exposure was associated with transient increases in transcripts for both of these genes. The kinetics of these increases in *fos* gene expression were similar to that obtained for members of the *Jun* gene family. Thus, activation of multiple *Jun* and *fos* genes could contribute to diverse nuclear signals in the response of cells to x-rays.

Treatment of HL-60 cells and other myeloid leukemia cells with TPA is associated with induction of the c-*Jun* gene. Similar effects were obtained in TPA-treated HL-205 cells. The response of these cells to TPA was associated with increases in c-*Jun* expression that were detectable at 6 hours and reached maximal levels by 24 hours. In contrast, similar exposures of HL-525 cells to TPA resulted in an increase in c-*Jun* expression which was transient at 12 hours and attenuated compared to that in the HL-205 link. These findings indicated that HL-525 cells are resistant at least in part to the effects of TPA on *Jun*/AP-1-mediated signaling events. Since TPA activates PKC and translocation of this enzyme is undetectable in HL-525 cells, the expression of PKC in the HL-205 and HL-525 lines was compared. HL-60 cells have been shown to express the α- and ß-PKC isozymes. Indeed, transcripts for PKCα and PKCß were detectable in HL-205 cells. However, constitutive expression of both genes was decreased by over 75% in HL-525 cells.

These results suggested that the relative resistance of HL-525 cells to TPA-induced c-*Jun* transcription could be attributable to low levels of PKC expression. Taken together with the finding that c-*Jun* expression is superinduced by ionizing radiation in HL-525 cells, these results also suggested that x-ray induced c-*Jun* expression may be mediated by events independent of PKCα and PKCß.

In order to further define the mechanisms responsible for induction of c-*Jun* expression in HL-525 cells, nuclear run-on assays were performed to determine the effects of x-rays on rates of c-*Jun* transcription. Similar studies were conducted in HL-205 cells for comparative purposes. The actin gene (positive control) was constitutively transcribed in HL-205 cells, while there was no detectable transcription of the ß-globin gene (negative control). Similar patterns were observed in HL-525

indicated that NAC is a specific inhibitor of x-ray-induced c-*Jun* transcription, presumably through its effects of ROIs.

Previous studies have demonstrated that the cellular response to other diverse classes of DNA-damaging agents, including ara-C, UV light, alkylating agents and etoposide, includes the induction of c-*Jun* expression. These findings have suggested that DNA damage per se is the signal responsible for activation of this gene. This response also appears to involve a protein kinase down-regulated by prolonged exposure to TPA. Since ROIs damage DNA, studies were performed to determine whether the response to these intermediates also includes a TPA-sensitive mechanism. Treatment of the HL-525 cells with TPA alone for 36-39 hours had no detectable effect on c-*Jun* mRNA levels. However, pretreatment with this agent blocked the x-ray induced increases in c-*Jun* expression by over 75%.

Similar studies were performed with bryostatin, an agent distinct from TPA which also transiently activates PKC. Treatment of HL-525 cells with bryostatin for 36 hours had little if any effect on the induction of c-*Jun* transcripts by ionizing radiation. Since H_2O_2 also acts as a DNA-damaging agent through the production of ROIs, similar experiments were performed in HL-525 cells treated with this agent. H_2O_2 transiently induced c-*Jun* expression in these cells and this effect was inhibited by NAC.

Pretreatment with TPA blocked H_2O_2-induced increases in c-*Jun* expression by 80%, while a similar exposure to bryostatin had no detectable effect. Taken together, these findings indicated that agents, such as ionizing radiation and H_2O_2 which produced ROIs, induce c-*Jun* expression by a mechanism down-regulated by TPA and not bryostatin.

NAC counteracts the effects of oxidative stress by scavenging ROIs and increasing intracellular glutathione (GSH). Previous studies have demonstrated that NAC is a potent inhibitor of phorbol ester-induced activation of the HIV-1 long terminal repeat. This antioxidant has also been found to inhibit activation of the nuclear factor κB (NF-κB) by phorbol esters and other agents such as H_2O_2. The available findings suggest that ROIs activate NF-κB by induced the release of the inhibitory subunit IκB. ROIs are also formed during the treatment of cells with ionizing radiation. Thus, the cellular response to this agent may involve the ROI-

also induced following x-ray exposure of the HL-525 variant, while treatment of these cells with TPA resulted in little if any effect on expression of these genes. These findings indicated that ionizing radiation increases *Jun/fos* expression through signaling pathways distinct from those activated during induction of these genes in TPA-treated cells.

The basis for the lack of PKC redistribution in TPA-treated HL-525 cells is unclear. Nonetheless, translocation of PKC from the cytosol to the cell membrane may be necessary for certain TPA-induced signaling events, such as induction of c-*Jun* expression. However, it is not known whether translocation to the cell membrane is necessary for activation of each of the different PKC isoforms. The present results demonstrate that prolonged treatment of the HL-525 variant with TPA blocks x-ray-induced increases in c-*Jun* expression. This finding lends support to the involvement of a PKC-dependent mechanism.

The HL-525 variant expresses relatively low levels of PKCα and PKCβ compared to HL-205 cells. Low to undetectable levels of PKCγ mRNA were also found in both the HL-205 and HL-525 lines (data not shown). Thus, other PKC isozymes which are sensitive to PKC down-regulation may be responsible for transducing signals which confer x-ray inducibility of the c-*Jun* gene. Alternatively, prolonged TPA treatment could cause down-regulation of other PKC-independent signaling pathways involved in induction of c-*Jun* by ionizing radiation. X-ray treatment was previously shown to be associated with activation of a PKC-like activity.

The following methods were used in Example 6.

Cell culture. Clone HL-205 was isolated from the human HL-60 myeloid leukemia cell line. The phorbol ester-resistant variant of HL-60 cells, designated HL-525, was isolated by exposing wild-type cells to low concentrations of 12-0-tetradecanoylphorbol-13-acetate (TPA; 0.5 to 3 nM) for 102 passages. These cells were maintained in RPMI 1640 medium containing 20% fetal bovine serum (FBS) with 1 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Irradiation was performed at room temperature using a Gamma cell 1000 (Atomic Energy at Canada Ltd., Ontario) with a ^{137}Cs source emitting at a fixed dose rate of 14.3 Gray (Gy)/min as determined by dosimetry.

EXAMPLE 7: Protocol for Treatment of Head and Neck Cancer with X-ray Induced TNF and Therapeutic X-rays

For treatment of patients with head and neck cancer, the following steps are followed:

1. Prepare a DNA molecule (genetic construct) comprising a radiation responsive enhancer-promoter operatively linked to an encoding region that encodes a polypeptide.

This construct preferaably comprises a CArG domain of an *Egr-1* promoter and the gene for the tumor necrosis factor. This construct is designated "construct A" for purposes of this example.

2. "Construct A" is put into a retrovirus that is self-inactivating.

3. Lymphokine activated killer (LAK) cells are infected with the retrovirus bearing "construct A." The cells are to be directed against the malignant cells in the head and neck.

4. The infected LAK cells are infused into the patient to be treated.

5. The head and neck region is irradiated.

The foregoing examples illustrate particular embodiments of the present invention. One of ordinary skill in the art will readily appreciate that changes, modifications and alterations to those embodiments can be made without departing from the true scope spirit of the invention.

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WHAT IS CLAIMED IS:

1. A synthetic DNA molecule comprising a radiation responsive enhancer-promoter operatively linked to an encoding region that encodes at least one polypeptide, which encoding region is operatively linked to a transcription-terminating region.

2. The DNA molecule according to claim 1 wherein said radiation responsive enhancer-promoter comprises a CArG domain of an *Egr-1* promoter, a tumor necrosis factor-α promoter or a c-*Jun* promoter.

3. The DNA molecule according to claim 1 wherein said encoding region encodes a polypeptide having the ability to interfere with the structural or functional integrity of a cell .

4. The DNA molecule according to claim 3 wherein said polypeptide is a cytokine, a dominant negative, a tumor suppressing factor, an angiogenesis inhibitor or a monocyte chemoattractant.

5. The DNA molecule according to claim 3 wherein said polypeptide is tumor necrosis factor-α, interleukin-4, JE, ricin, PF4 Pseudomonas toxin, p53, the retinoblastoma gene product or the Wilms' tumor gene product.

6. The DNA molecule according to claim 3 wherein said polypeptide protects a normal cell from the pathological effects of radiation.

7. The DNA molecule according to claim 6 wherein said polypeptide is interleukin-1, interleukin-6, tumor necrosis factor-α, a tissue growth factor, a tissue growth factor receptor or a free radical scavenger.

18. The DNA molecule according to claim 17 wherein said encoding region comprises:

(a) a first encoding sequence that encodes a DNA binding domain of a first transcription factor;

(b) a second encoding sequence that encodes an activation or repression domain of a second transcription factor;

(c) a third encoding sequence that encodes a nuclear localization signal, whereby the first, second and third encoding sequences are operatively linked in frame to each other in any order with the proviso that said third encoding sequence need be present only if said first or second encoding sequence does not encode a nuclear localization signal; and

(d) a transcription-terminating region that is operatively linked to any of the first, second or third encoding sequences such that the transcription-terminating region is located 3' to all of the first, second and third encoding sequences.

19. The DNA molecule according to claim 18 wherein said first transcription factor is GAL4.

20. The DNA molecule according to claim 18 wherein said second encoding sequence encodes the VP-16 or NF-κB activation domain.

21. The DNA molecule according to claim 18 wherein said second encoding sequence encodes the repression domain of the Wilms' tumor suppressor gene WT1 or the repression domain of *Egr-1*.

22. An isolated and purified DNA molecule comprising a binding region that is capable of binding a DNA binding domain of a transcription factor, which binding region is operatively linked to a minimal promoter that is operatively linked to an

31. The DNA molecule according to claim 30 wherein said polypeptide is plasminogen activator, a plasminogen activator inhibitor or a streptokinase.

32. The DNA molecule according to claim 22 wherein said polypeptide catalyses the conversion of a pro-drug to a drug.

33. The DNA molecule according to claim 32 wherein said polypeptide is herpes simplex virus thymidine kinase or a cytosine deaminase.

34. The DNA molecule according to claim 22 wherein said polypeptide is a surface antigen product of a major histocompatibility complex gene.

35. The DNA molecule according to claim 34 wherein said antigen product is HLA-A, HLA-B or HLA-C.

36. A pharmaceutical composition comprising a physiologically acceptable carrier and a DNA molecule of claim 1.

37. A pharmaceutical composition comprising a physiologically acceptable carrier and a DNA molecule according to claim 22.

38. A cell transformed or transfected with a DNA molecule comprising a radiation responsive enhancer-promoter operatively linked to an encoding region that encodes at least one polypeptide, which encoding region is operatively linked to a transcription-terminating region.

39. The cell according to claim 38 further transformed or transfected with a DNA molecule comprising a binding region that is capable of binding said DNA binding domain of said first transcription factor, which binding region is operatively linked to a minimal promoter that is operatively linked to an encoding region that encodes a polypeptide, which encoding region is operatively linked to a transcription-terminating region.

(d) a transcription-terminating region that is operatively linked to any of the first, second or third encoding sequences such that the transcription-terminating region is located 3' to all of the first, second and third encoding sequences.

45. A process of regulating the expression of a polypeptide comprising the steps of:

(a) operatively linking a radiation responsive enhancer-promoter to an encoding region that comprises:

(i) a first encoding sequence that encodes a DNA binding domain of a first transcription factor;

(ii) a second encoding sequence that encodes an activation or repression domain of a second transcription factor;

(iii) a third encoding sequence that encodes a nuclear localization signal, whereby the first, second and third encoding sequences are operatively linked in frame to each other in any order with the proviso that said third encoding sequence need be present only if said first or second encoding sequence does not encode a nuclear localization signal; and

(iv) a transcription-terminating region that is operatively linked to any of the first, second or third encoding sequences such that the transcription-terminating region is located 3' to all of the first, second and third encoding sequences to form a first DNA molecule; and

(b) operatively linking a binding region that is capable of binding said DNA binding domain of said first transcription factor to a minimal promoter that is operatively linked to an encoding region that encodes a polypeptide, which encoding

need be present only if said first or second encoding sequence does not encode a nuclear localization signal; and

(iv) a transcription-terminating region that is operatively linked to any of the first, second or third encoding sequences such that the transcription-terminating region is located 3' to all of the first, second and third encoding sequences; and

(2) a second DNA molecule comprising a binding region that is capable of binding said DNA binding domain of said first transcription factor, which binding region is operatively linked to a minimal promoter that is operatively linked to an encoding region that encodes a polypeptide having tumor cell cytotoxic activity, which encoding region is operatively linked to a transcription-terminating region; and

(b) exposing said cell to an effective expression-inducing dose of ionizing radiation.

INTERNATIONAL SEARCH REPORT

Inte. .onal Application No
PCT/US 93/08432

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/67 C12N15/62 C12N15/85 A61K31/70 A61K41/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 11033 (ARCH DEVELOPMENT CORPORATION) 9 July 1992 cited in the application see page 3, line 30 - page 5, line 4 see page 5, line 6 - page 7, line 31 see page 10, line 18 - page 12, line 8 see page 30, line 19 - page 32, line 14 ---	1-7, 9-11,17, 36-39
X	PROC. NATL. ACAD SCI. vol. 88, no. 6 , 15 March 1991 , NATL. ACAD SCI., WASHINGTON, DC, US; pages 2156 - 2160 D.E. HALLAHAN ET AL. 'Protein kinase C mediates x-ray inducibility of nuclear signal transducers EGR1 and JUN' cited in the application see page 2156, left column, line 1 - line 20 ---	1,2
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 November 1993	17 -12- 1993

Name and mailing address of the ISA	Authorized officer
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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Ina ional Applicanon No
PCT/US 93/08432

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9211033	09-07-92	AU-A-	9170291	22-07-92
		EP-A-	0563278	06-10-93